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1 Screening for Th17-dependent pneumococcal vaccine antigens: comparison of murine
2 and human cellular immune responses

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Abstract

Conjugate vaccines against *Streptococcus pneumoniae* have significantly reduced diseases caused by serotypes included in those vaccines; however, there is still need for vaccines that confer serotype-independent protection. In the current study, we have constructed a library of conserved surface proteins from *S. pneumoniae* and screened for IL-17A and IL-22 production in human immune cells obtained from adenoidal/tonsillar tissue of children and IL-17A production from splenocytes from mice that were immunized with a killed whole cell vaccine or previously exposed to pneumococcus. A positive correlation was found between rankings of proteins from human IL-17A and IL-22 screens, but not between human and mouse screens. All proteins were tested for protection against colonization, from which we identified protective antigens that are IL-17A dependent. We found that the likelihood of finding a protective antigen is significantly higher from groups of proteins ranked in the top 50% of all three screens than for groups of proteins ranked in the bottom 50% of all three. The results thus confirmed the value of such screens to identify Th17 antigens. Further, these experiments have evaluated and compared the breadth of human and mouse Th17 responses to pneumococcal colonization and enabled the identification of potential vaccine candidates based on immunological responses in mouse and human cells.

Introduction

Streptococcus pneumoniae, or pneumococcus, is a Gram-positive bacterium that is a major cause of morbidity and mortality in infants, toddlers and the elderly in both developed and developing countries. Introduction of the first conjugate vaccine PCV7, followed by PCV10 and PCV13, has greatly reduced the incidence of invasive disease and colonization caused by the serotypes included in these vaccines (1). Despite the success of these vaccines, serotypes that are not included in these vaccines pose an emerging threat. Indeed, there are at least 97 identified pneumococcal serotypes with variability in regional distribution of predominant serotypes; furthermore, the phenomenon of serotype replacement has reduced the impact of these vaccines in many settings (2).

While it is well established that anticapsular antibodies are sufficient to prevent invasive disease such as pneumonia, meningitis and sepsis, other mechanisms of immunity may also play important roles. Either live exposure to pneumococcus or immunization with killed whole cell vaccine (SPWCV) can induce CD4⁺ Th17 dependent protection against nasopharyngeal colonization in mice (3-5). The reduction in colonization in mice is dependent on generating pneumococcus-specific Th17 immunity and recruiting neutrophils to the mucosal site (3).

Evidence is accumulating to suggest that Th17-based immunity is also important for protection against pneumococcus in humans. The existence of pneumococcal-specific Th17-type T cells was demonstrated in both children and adults by analysing Th17 responses to a killed preparation of pneumococcus (pneumococcal whole cell antigen, WCA) or individual pneumococcal antigens (6). Furthermore, the frequency of pneumococcal-specific Th1 and Th17 CD4⁺ T cells within mucosal lymphoid tissue demonstrates age-dependent increases, likely due to cumulative exposure to pneumococcus (7). In an experimental human challenge model, lung IL-

17A-secreting CD4⁺ memory T-cells were detected following intentional pneumococcal carriage (8). Furthermore, patients with autosomal dominant hyper-IgE syndrome (Job's syndrome), who lack the ability to generate memory Th17 cells due to mutations in the *stat3* gene, are highly susceptible to recurrent pneumococcal infections (9, 10). More recently two studies provided support for a role of IL-17A in pneumococcal carriage in children: a specific polymorphism of the IL-17 gene (G-152A) in Finnish children (11) and decreased IL-17A secretion in Fijian children (12) were associated with increased risk of pneumococcal carriage.

In addition to Th17 cells, antibodies are also likely to play an important role in protection against pneumococcal disease. A SPWCV consisting of WCA and aluminium adjuvant given subcutaneously or intramuscularly elicits anti-protein antibody-mediated protection against pneumococcal pneumonia and sepsis in mice, which can also be reproduced by passive transfer of antibodies obtained from rabbits immunized with SPWCV (13). Naturally acquired protection against pneumococcal disease in humans was recently shown to depend largely on antibody to protein antigens rather than to capsular polysaccharides (14).

As a consequence, there has been a concerted effort over many years to identify protective protein antigens against pneumococcal disease (15, 16). Traditional methods, such as protein separation by two-dimensional gel electrophoresis and identification by mass spectroscopy, have been used to identify proteins from the cell wall fraction of pneumococcus that interact with convalescent sera from patients (17). As another approach, libraries of purified surface proteins have been used to identify antigens that may induce protection against pneumococcal invasive disease (18). A display library expressing 15-150 amino acid fragments of the pneumococcal proteome was used to identify proteins that interact with serum from infected individuals, leading to selection of StkP and PcsB as candidate antigens (19).

Using similar approaches, other investigators have used pneumococcal antigens to identify potentially protective T-cell antigens. Putative Th17-eliciting antigens were identified from the soluble fraction of the WCA using preparative SDS gel electrophoresis followed by mass spectroscopy (20). In a more comprehensive approach using the ATLAS© system, an expression library containing >96% of predicted pneumococcal proteins was used to identify antigens recognized by Th17 cells from SPWCV-immunized mice (21) and from human volunteers naturally exposed to pneumococcus (22).

The studies cited above used either mouse splenocytes or human peripheral blood mononuclear cells (PBMCs) as a source of immune cells. There have been some studies using arguably a more relevant source of immune cells, the human mucosal lymphoid tissue, probed for Th17 responses following stimulation with a small number of pneumococcal proteins (23-25), but a more comprehensive analysis of the range of Th17 responses to the pneumococcal proteome has not been performed. Here, we constructed a protein library consisting of genetically conserved pneumococcal surface proteins. We used this library to screen human adenoidal cells for Th17-cytokine-inducing antigens and compared these human responses to those observed from splenocytes from mice immunized with SPWCV or exposed to pneumococcus. Positive correlations between two human screens but no correlations between human and murine screens were observed. Several antigens were identified that subsequently showed IL-17A-dependent protection against pneumococcal colonization in mice.

Results

Age-dependent immune response to WCA

We obtained 35 adenoidal samples from children undergoing adenoidectomy and analyzed IL-17A and IL-22 production in response to stimulation with WCA in 33 of these

119 samples. As shown in Figure 1, there was an age-dependent, significant increase in IL-
120 17A responses ($p=0.0235$, $r=0.3996$) and IL-22 ($p=0.0429$, $r=0.3786$). The finding that
121 immune responses to pneumococcus in adenoids in children tend to increase with age
122 supports the hypothesis that these adenoidal cells may represent a good source of
123 responsive immune cells for antigen discovery.

124 **Screening antigens using human adenoidal cells**

125 Fifty-six recombinant proteins out of 81 genetic constructs were successfully expressed
126 in *E. coli* and purified using Ni-NTA chromatography. These proteins were further
127 purified by gel filtration before being used in stimulation experiments with adenoidal cells.
128 Due to the limited number of cells obtained from each adenoid, we were able to screen
129 all 56 proteins in 13 samples and only a subgroup of these proteins with the other 22
130 human samples. Both we and others have showed that IL-17A production in human in
131 response to pneumococcal whole cell or purified pneumococcal proteins are mostly due
132 to stimulation of memory T cells (26, 27). The results of IL-17A and IL-22 production in
133 these 13 samples after subtracting the baseline responses following incubation with
134 medium alone are shown in Figure 2. IL-17A responses from each protein were
135 generally in the range of 50-100 pg/ml (mean 157 pg/ml), with WCA having the highest
136 responses (far right) (Figure 2A). IL-22 responses were generally higher than IL-17A,
137 mostly ranging from 200-600 pg/ml (Figure 2B).

138 **Screening antigens using murine immune cells**

139 While colonization and infection models in mice are widely used to test candidate
140 pneumococcal vaccines, the extent to which murine immune responses mimic human
141 responses is still unclear. A comparison of the responses of murine and human immune
142 cells following stimulation with proteins from the same pneumococcal library may
143 improve our understanding of the differences between and common features of these
144 two species. To this end, murine splenocytes were obtained from mice either

immunized with SPWCV or previously colonized with a serotype 6B pneumococcal strain for 4 weeks (the latter model may more closely mimic pneumococcal exposure in humans). Both methods result in the generation of protective and antigen-specific memory CD4⁺ T cell responses against nasopharyngeal colonization in mice (3, 13, 28, 29), and we have confirmed that these mice were protected from nasal colonization (data not shown). IL-17A responses following stimulation of splenocytes from these two mice models with the library of proteins are shown in Figure 3. Stimulation with SPWCA and proteins in splenocytes from naïve mice did not generate any IL-17A (data not shown). Responses in SPWCV immunized mice, most of which were between 50 and 200 pg/ml (Figure 3A) were generally lower than those in pneumococcus-exposed mice, most of which were between 100 and 500 pg/ml (Figure 3B). SPWCA induced the largest responses in both mouse models (far right data points).

Evaluation of antigens in colonization models. Next, we tested the ability of the library of proteins to provide protection against colonization in mice when used as immunogens. Mice were immunized intranasally twice at a one-week interval with one of the top 5 murine proteins (10 µg/dose) and 1 µg of CT; control mice received 1 µg of CT alone. The whole library was tested in six different experiments, as shown in Figure 4. A total of 18 constructs (16 antigens) was found to be protective in this model; their identities and predicted functions are listed in table 1.

Protection against colonization is IL-17A dependent

We have shown previously that protection against colonization is dependent on CD4⁺ Th17 cells whereas antibodies directed against antigens contained in the whole cell vaccine were not protective in the colonization model (3, 13, 28). We then tested whether the protection afforded by these antigens is dependent on IL-17A production. To facilitate the experiment, we chose combinations of the three antigens which induced greatest responses either in murine screens (SP0648-3, SP0757 and SP1500) or in the

human IL-17A screen (SP0648-1, SP0662-1 and SP0742) and tested the dependence of protection on IL-17A. Mice were immunized intranasally and were given either anti-IL17A antibody or an isotype control antibody both one day before and 3 days after challenge inoculation with *S. pneumoniae*. As shown in Figure 5, immunization with both antigen combinations (Figure 5A & B) protected mice from colonization while protection was abolished in immunized mice treated with antibody directed against IL-17A, thus confirming the role of this cytokine in this model.

Ranking of antigens and correlation between screens

Proteins were ranked in each screen as explained in the methods section. We compared the ranking results of SPWCV-immunized mice, pneumococcal exposed mice and human samples with respect to IL-17A and IL-22. There was a weak correlation between the results from immune cells in the two different mouse models (Figure S1A) while there was a strong positive correlation between the IL-17A and IL-22 screens using human cells (Figure 6). No statistically significant correlation was found between the IL-17A screen results using human cells and cells from SPWCV-immunized mice (Figure S1B) or mice previously exposed to pneumococcus (Figure S1C), nor was any correlation between human cell IL-22 production and either mouse screen found (data not shown).

Validation of the screening method

We analyzed how well each screen predicted protection against colonization. Overall, the rate of protection among the proteins in the library is quite high (18/56, 32.1%; Figure 4) comparing to regular screening with whole genome (21), which probably reflects the way the library was designed. Antigens were categorized as belonging either to the top or bottom 50%, based on their rankings in each screen. We then compared the “hit rate” (i.e. identifying an antigen that is protective in the colonization model) in the top 50% responders versus the bottom 50% responders in the two murine and one human IL-17A

197 screens. While the hit rates were higher in the top 50% responders, none of these
198 differences was significant (table 2). In contrast, when we identified antigens that were
199 highly ranked either in two screens or amongst all three screens, the hit rates were
200 significantly higher: for example, for antigens that were in the top 50% rank in all three
201 screens, the hit rate was 61.5%, significantly higher than the 11.1% hit rate for antigens
202 that fall into the bottom 50% in all three screens ($p=0.0306$ by Fisher's exact test), and
203 about twice the hit rate for the whole library, which trended towards significance ($p=0.06$,
204 by Fisher's). When the same analysis was done with two murine and human IL-22
205 screen, a similar result was obtained. The hit rate in the top 50% rank in all three
206 screens was 58.5%, comparing to 8.3% in the bottom of all three screens ($p=0.009$).

207 Discussion

208 Using a conserved pneumococcal surface protein library, we stimulated human
209 and murine immune cells in order to identify antigens that elicit IL-17A and IL-22
210 responses and tested which of them confer protection against pneumococcal
211 colonization following intranasal immunization in mice. Overall, we found that antigens
212 that more consistently induced cytokine responses across both murine and human cell
213 screens were more likely to be protective. In addition, we identified many antigens that
214 showed IL-17A-dependent protection against pneumococcal colonization in mice.

215 One goal of this study was to investigate whether the immune responses to
216 pneumococcus in murine and human cells are correlated and whether murine screens
217 could predict the results of the human screen. IL-17A and IL-22 responses of human
218 cells were highly correlated to each other, which confirms our previous findings that IL-
219 17A and IL-22 responses to WCA in children's PBMC are highly correlated (6). However,
220 there was no correlation between the rankings of the IL-17A responses to our protein
221 library in murine and human screens, suggesting that there are differences in responses
222 that are both host- (mouse vs. human) and exposure-dependent (live vs. killed bacteria).

The lack of correlation between exposed mouse and human cells could be explained by many factors. First, there are clear differences between mouse and human immune systems (30). Furthermore, the local environment for pneumococcal colonization might not be the same in mice and human, given growing evidence of the influence of microbiota on immune development (31). Thirdly, most children may have been exposed to pneumococcus many times, after which they may have generated high Th17 response to nonprotective proteins whereas the mice were only exposed to pneumococcus or pneumococcal antigens for a short defined period in our experiment. Lastly, in addition to intrinsic differences between mouse and human immune cells, we are comparing human adenoidal mucosal cells to murine splenocytes, cells from very different compartments.

Despite these differences, we were able to identify several protective candidates by combining the three screens, which suggests that screening for cytokine production using cells from different sources may be a useful method to identify cytokine-mediated protection. Indeed, we found that the protective hit rate was highest in the pool of proteins that induced higher responses in all the screens. The 16 protective antigens that we identified have not previously been reported as conferring protection, with the single exception of SP1683 which was recently reported as a Th17-dependent antigen which protects against colonization while this manuscript was being prepared (32). While protection against colonization by any single antigen did not approach that of SPWCV in mouse models (Figure 4), a combination of three antigens significantly improved the protective efficacy of any individual protein antigen (Figure 5). This suggests that a candidate protein-based vaccine should likely comprise several antigens, to maximize protection and coverage.

Another important implication of our work is that immune responses demonstrable in mouse models do not accurately predict those of humans. In light of

the recent failure of at least two different protein vaccine formulations(33, 34) to provide protection against colonization, one is left wondering whether excessive reliance on mouse models may be responsible. Clearly, murine models are much more convenient and practical for large scale screening and identification of potential candidates, as were performed here. However, prior to performing expensive and time-consuming proof-of-concept studies of impact on colonization in toddler or infant subjects, perhaps other approaches that can serve to de-risk the process may be useful. One approach would be to try to establish correlates of protection, by performing longitudinal studies of colonization and systemic T cell responses in children. Another approach may be to test promising candidates in intentional challenge studies in humans (8, 35). A potential caveat to this strategy is that intentional challenge studies are performed in adults, whereas the intended target population of these vaccines is generally toddlers and infants. However, such studies could still be helpful in providing a gating strategy: impact on carriage density or duration of carriage in a properly powered study of intentionally challenged adult volunteers could be used to decide whether or not to pursue studies of the candidate vaccine in younger subjects.

A potential limitation of our study is that children undergoing adenoidectomy, who are the only practical and ethically acceptable source of pediatric NALT, may be immunologically distinct from a randomly selected sample of healthy children. Nevertheless, previous studies have confirmed a very similar pattern of acquisition of serum antibodies with age in both these children and healthy controls. Furthermore, rates of pneumococcal colonization at the time of surgery are also similar to those seen in healthy children of comparable age (36). Another potential limitation is that some children may have had limited prior pneumococcal exposure at the time of surgery. However previous studies have shown that the majority of such children have mucosal specific immune responses to pneumococcal protein antigens (37).

In summary, we report here the results of screening of human and mouse cells following exposure to pneumococcus using a library of conserved pneumococcal proteins. We believe the use of human and murine cells for this type of screening can inform the selection of potential candidates worthy of further study. This approach could also be applied to the identification of other important mucosal pathogens whose route of entry begins in the nasopharynx.

Materials and Methods:

Materials: Cholera toxin was purchased from List Biological Laboratories. Ni-NTA resin was purchased from Qiagen. CloneEZ PCR cloning kits were obtained from Genscript Inc. All other reagents were obtained from Sigma or Thermo Fisher Scientific.

Selection of protein candidates by bioinformatic analysis *in silico*

We chose 42 *S. pneumoniae* sequences (including some finished and others in draft form) from the integrated microbial genomes website (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). Beginning with the TIGR4 strain, we identified 335 proteins with a secretion signal peptide and 15 proteins with possible cell wall anchor motifs. The protein library was then narrowed down to 76 proteins based on the following parameters chosen *a priori*:

- a. Conservation across all 42 sequences defined as >90% identity at the amino acid level (reduced to 203).
- b. Exclusion of proteins with >40% homology with proteins in the human genome (reduced to 160).
- c. Exclusion of proteins containing an extracellular domain smaller than 100 amino acids (to focus on proteins more likely to be accessible to antibodies in the presence of polysaccharide capsule; reduced to 88)

We specifically excluded previously-studied antigens (including PsaA (38), SP2018 and SP0148 (21), StkP and PcsB (19), Pht family proteins (39)) in order to focus on novel

antigens. The breakdown of the 76 proteins (see table S1) is as follows: 23 hypothetical proteins, 17 proteins proposed to play roles in substrate binding and transportation, 17 proteins with predicted enzymatic activity and 19 others with unknown or hypothetical functions. Only extracellular domains without signal peptides or transmembrane regions were cloned.

Construction of the pneumococcal expression library.

The extracellular domains of selected proteins were amplified by PCR using TIGR4 genomic DNA as template and then integrated into pET21b expression vectors using the CloneEZ PCR cloning kit. Two large (>250 kDa) proteins (SP0648 and SP1154) proved difficult to purify full-length. We divided each amino acid sequence into 3 parts based on predictions of their secondary structure by BCL::Jufo (http://meilerlab.org/index.php/servers/show?s_id=5), making truncations in unconserved sequence areas, and purified each fragment separately. The two possible extracellular domains of one protein, SP0662, were both cloned and designated SP0662-1 and SP0662-2. Thus the final protein library consists of 81 proteins and peptides. Plasmid inserts were sequenced by Genewiz Inc. for confirmation.

Protein Purification. *E. coli* transformants containing the cloned proteins were grown to OD₆₀₀=0.6 and protein expression induced overnight with 0.2 mM IPTG at 16°C. Cells were spun down and pellets resuspended in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH8.0) and lysed by sonication. The proteins of interest were purified from supernatants over a Ni-NTA column and eluted in imidazole buffer. Elutions containing each protein were combined and purified over a gel-filtration column in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0.

Stimulation of mouse immune cells and human adenoidal cells

Splenocytes were harvested from mice that were either immunized with SPWCV intranasally or colonized intranasally with *S. pneumoniae* strain 0603 (6B serotype) (29).

Stimulations were carried out for 3 days with 5 µg/ml of each protein at 37°C with 5% CO₂. Plates were then spun to pellet the cells, after which the supernatants were collected and assayed for IL-17A using a mouse IL-17A ELISA kit (R&D Systems, Inc). Human adenoidal mononuclear cells from children undergoing adenoidectomy were separated on Ficoll gradients and cultured at 1x10⁶ cells per well in 48 well plates. Cells were stimulated with 8 µg/ml of recombinant proteins or SPWCA at a concentration representing 1x10⁶ cfu/ml of killed bacteria for 7 days and supernatants assayed for IL-17A and IL-22 concentrations using human IL-17A and IL-22 ELISA kits (eBioscience).

Ranking of antigens. Cytokine responses to each protein were ranked based on the average of the rankings among all responses to all the proteins in the cells from each donor (child or mouse) in each screen, instead of their actual values, to minimize the effects of variation in the responsiveness of the cells of each individual. In each experiment, the protein with the greatest cytokine response was allocated the highest rank and each subsequent protein was assigned a chronological rank in the decreasing order of their responses. Proteins that had responses lower than that of unstimulated cells were all numbered “1”. The ranking numbers from each experiment were averaged to calculate the final rank of each protein.

Immunization and challenge of mice. Female C57BL/6J mice (Jackson lab) were used in all experiments. All animal studies were approved by our local animal ethics committee. The age at time of first immunization was 4-6 weeks. Intranasal (i.n.) immunization was done by instilling 20 µl of saline with 1 µg of cholera toxin (CT) as a control, or CT mixed with 10 µg of antigen as specified, atraumatically into unanesthetized mice twice at a one-week interval. Blood was drawn 3 weeks after the last immunization, and assayed for IL-17A production upon stimulation with 5 µg/ml of the corresponding protein for 6 days. Nasopharyngeal colonization with the clinical pneumococcal isolate 0603 (serotype 6B) was carried out as previously described (29).

IL-17A depletion. Anti-IL-17A monoclonal (clone 17F3) and matching isotype-control antibodies were purchased from BioXcell. Mice were injected intraperitoneally with a dose of 150 µg per mouse 24 hours before and 3 days after infection.

Statistical analysis. Correlation was analyzed by the nonparametric Spearman method. NP colonization densities were compared by the Mann-Whitney *U* test. Both were done using PRISM (version 7.0a, GraphPad Software, Inc).

360

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Table 1 Protective antigens against pneumococcal colonization

	Gene name	Annotation
1	SP0321 ^b	PTS system, IIA component
2	SP0648 ^{a,b,c,e}	Beta-galactosidase
3	SP0662 ^{a,b,c}	Sensor histidine kinase
4	SP0742 ^a	Hypothetical protein
5	SP0757 ^{a,b,c}	Cell division protein FtsX
6	SP0785 ^{a,b,c}	Hypothetical protein
7	SP0787 ^{a,c}	Hypothetical protein
8	SP0878 ^b	SpoE family protein
9	SP0899 ^b	Hypothetical protein
10	SP1032 ^{a,b,c}	Iron-compound ABC transporter
11	SP1069 ^d	Hypothetical protein
12	SP1154-2 ^a	IgA1-specific metallopeptidase
13	SP1386 ^{a,c}	Spermidine/putrescine ABC transporter
14	SP1479 ^b	Peptidoglycan N-acetylglucosamine deacetylase A
15	SP1500 ^{b,c}	Amino acid ABC transporter substrate-binding protein
16	SP1683 ^{a,b,c}	Carbohydrate ABC transporter substrate-binding protein

a, top 50% in human IL-17A screen; b, top 50% in murine WCV screen; c, top 50% in exposed murine screen; d, not in any top 50% screen, e, SP0648 consisted of three separate constructs, all of which were in the top 50% of all screens.

Table 2 Hit rate in the top 50% responders in different screens

Screens	Hit rate in top 50%	Hit rate in bottom 50%
All three	8/13 (61.5%)	1/9 (11.1%)
Human/WCV	8/14 (57.1%)	1/13 (7.7%)
Human/Exposed	10/21 (47.6%)	4/18 (22.2%)
WCV/Exposed	9/16 (56.3%)	3/17 (17.6%)
Human	12/27 (44.4%)	6/27 (22.2%)
WCV	12/28 (42.9%)	6/28 (21.4%)
Exposed	11/28 (39.3%)	7/28 (25%)

Top 50% responders from ranked screen results of immunized mice (WCV), exposed mice (exposed) and human IL-17A (human). Each screen was ranked and then the top 50% or bottom 50% of proteins in the rankings were selected and overlap between these antigens was shown here.

Figure legends

Figure 1. IL-17A and IL-22 responses to SPWCV are positively correlated with donors' ages. Adenoidal cells were stimulated with SPWCV and IL-17A and IL-22 were measured from the cell supernatant. The correlations were evaluated using the Spearman test.

Figure 2. Responses of human adenoidal cells to stimulation with each protein or with whole cell vaccine. Human adenoidal mononuclear cells were stimulated with 8 µg/ml of recombinant proteins or SPWCA at a concentration representing 1×10^6 cfu/ml of killed bacteria for 7 days and cytokines were measured with ELISA. The graph is presented as mean±SEM. The antigens used for screening are: SP0079, SP0084, SP0092, SP0098, SP0127, SP0149, SP0191, SP0198, SP0249, SP0346, SP0435, SP0453, SP0564, SP0582, SP0601, SP0604, SP0617, SP0620, SP0629, SP0648-1, SP0648-2, SP0648-3, SP0659, SP0662-1, SP0662-2, SP0678, SP0724, SP0742, SP0757, SP0785, SP0787, SP0878, SP0899, SP1002, SP1032, SP1069, SP1154-2, SP1386, SP1404, SP1479, SP1500, SP1534, SP1545, SP1560, SP1652, SP1683, SP1826, SP1872, SP1942, SP2070, SP2083, SP2145, SP2192, SP2197, SP2207, SP2218 and SPWCV (from left to right). SP0321 was toxic to human cells and was not used in human screens.

Figure 3. Responses of murine splenocytes to stimulation with each protein or with whole cell vaccine. Mice splenocytes were stimulated with 5 µg/ml of recombinant proteins or SPWCA at a concentration representing 1×10^6 cfu/ml of killed bacteria for 3 days and IL-17A was measured with ELISA. The graph is presented as mean±SEM. The antigens used for screening are: SP0079, SP0084, SP0092, SP0098, SP0127, SP0149, SP0191, SP0198, SP0249, SP0321, SP0346, SP0435, SP0453, SP0564, SP0582, SP0601, SP0604, SP0617, SP0620, SP0629, SP0648-1, SP0648-2, SP0648-3, SP0659, SP0662-1, SP0662-2, SP0678, SP0724, SP0742, SP0757, SP0785, SP0787, SP0878, SP0899, SP1002, SP1032, SP1069, SP1154-2, SP1386, SP1404, SP1479, SP1500,

SP1534, SP1545, SP1560, SP1652, SP1683, SP1826, SP1872, SP1942, SP2070, SP2083, SP2145, SP2192, SP2197, SP2207, SP2218 and SPWCV (from left to right). (A) IL-17A levels following stimulation of splenocytes from SPWCV immunized mice. (B) IL-17A levels following stimulation of splenocytes from pneumococcus-exposed mice.

Figure 4. Protection against colonization by antigens from the surface protein library. Mice were immunized with 10 µg of each protein and 1 µg of cholera toxin twice weekly and challenged 4 weeks after the last immunization with a serotype 6B clinical strain. Nasal wash was collected 7 days later and bacterial CFU was determined by plating. Each dot represents CFU recovered from one mouse and the lines for each column represent geometric means. Colonization rates in mice receiving antigen plus adjuvant CT immunization were compared to those in control mice (receiving CT alone) using the unpaired nonparametric Mann-Whitney test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 5. Protection against nasal colonization conferred by proteins is IL-17A dependent. Mice were immunized with top-ranked antigens identified via the human screen (SP0648-1, SP0662-1 and SP0742) or via the mouse screen (SP0648-3, SP0757 and SP1500) intranasally. Mice were immunized with 10 µg of each protein and 1 µg of cholera toxin twice weekly and challenged 4 weeks after the last immunization with a serotype 6B clinical strain. Mice received either anti-IL-17A or isotype control antibody one day prior to and 3 days after the infection. Statistical analysis was performed with unpaired nonparametric Mann-Whitney test. n.s, not significant; *, $p < 0.05$; ****, $p < 0.0001$.

Figure 6. Correlation between human IL-17A and IL-22 screens. The ranking of each antigen was analyzed for their correlation between each screen using nonparametric Spearman method.

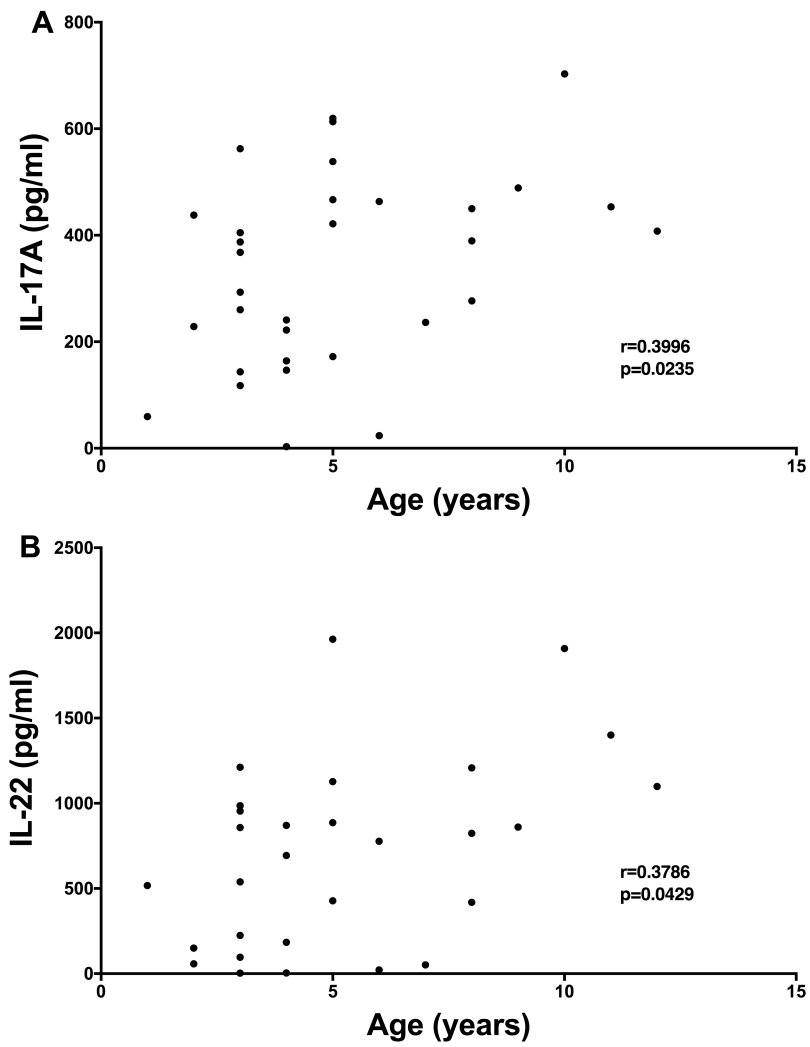


Figure 1

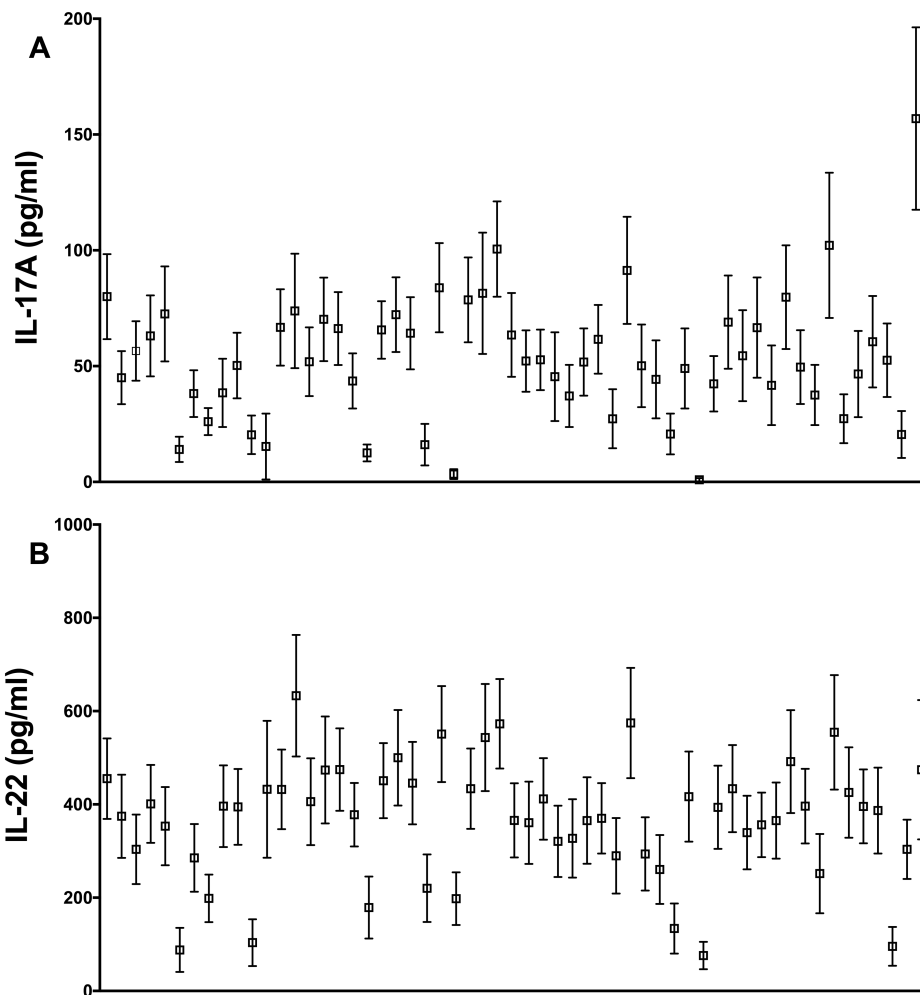


Figure 2

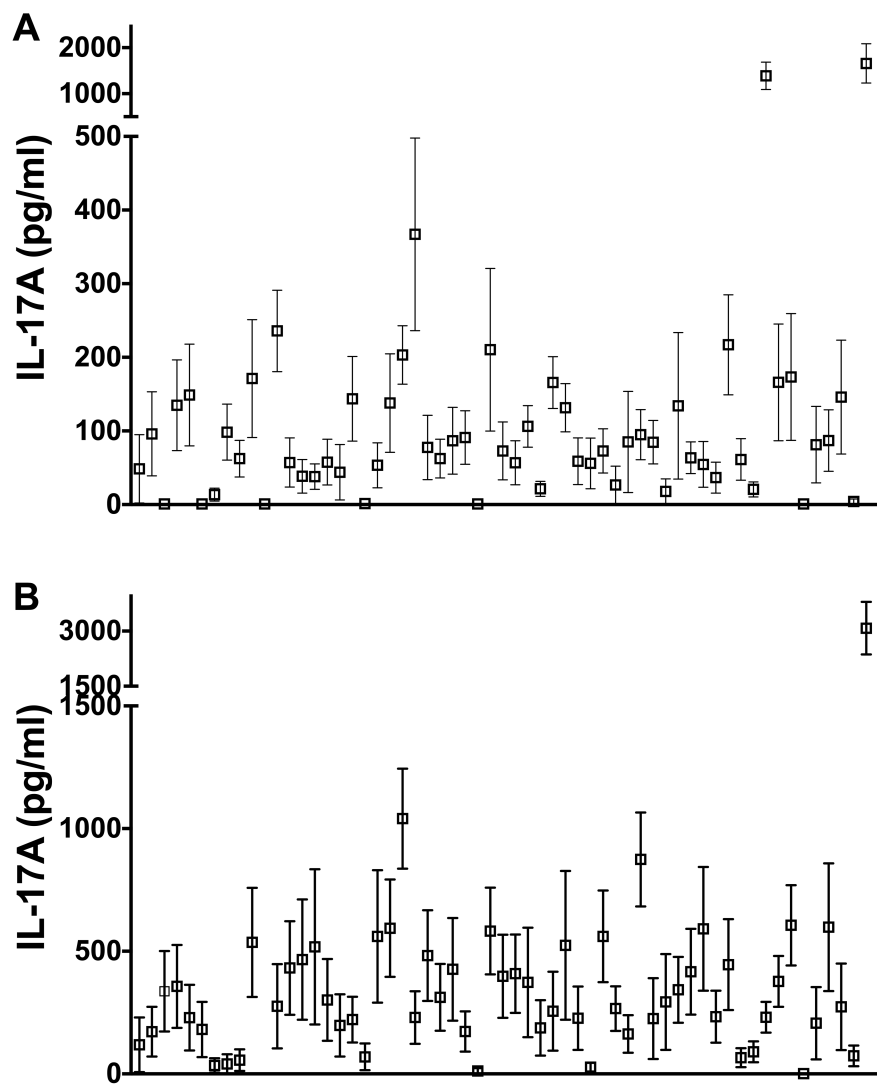


Figure 3

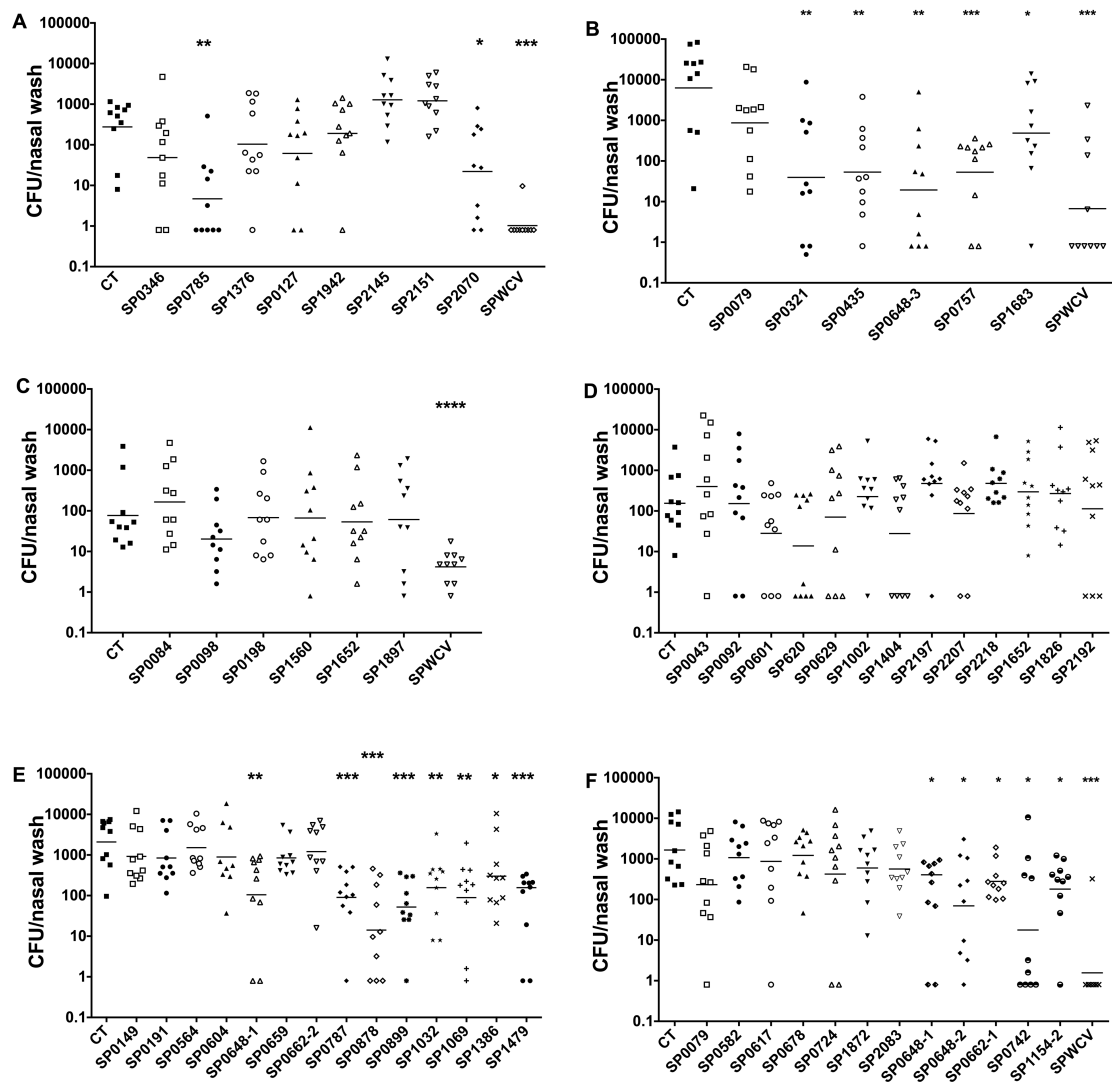


Figure 4

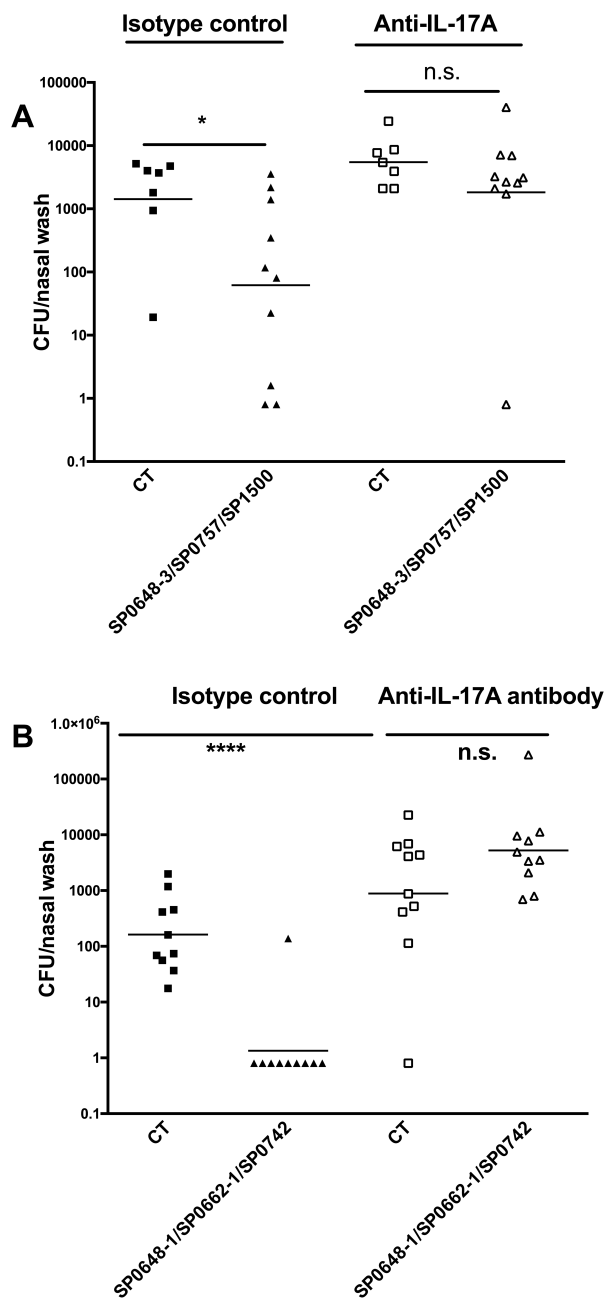


Figure 5

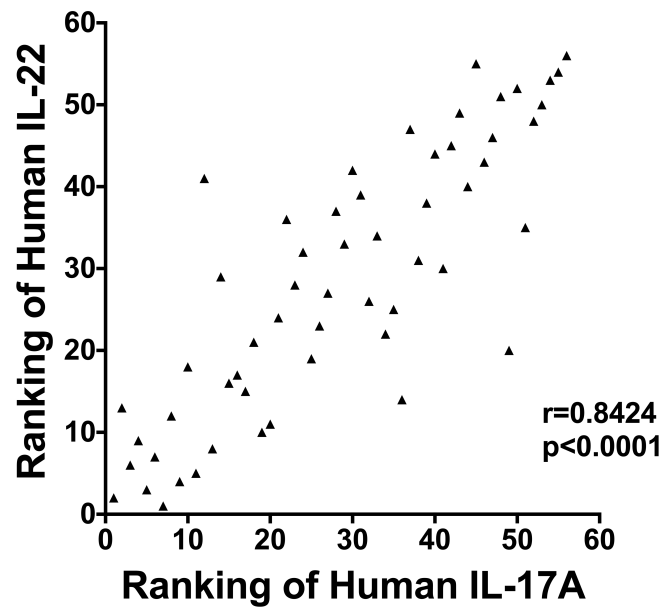


Figure 6